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14. ABSTRACT: Altered nucleotide excision repair (NER) activity may be a common, initial and detectable event leading to genomic instability in human breast epithelial cells, ductal carcinoma in situ or early invasive breast cancers. Our objective is to study DNA repair activity in primary breast epithelial cells and cancer tissues from women at risk for or diagnosed with breast cancer to determine if NER activity can be reliably and practically quantitated from these tissues and cells. The three specific aims are: 1) Apply a quantitative local DNA damage immunofluorescent assay to measure NER activity in single cells derived from women at risk for or with breast cancer, from ductal lavage or MRI directed biopsy samples of breast epithelial cells or tumor cells, and from appropriate controls. 2) Develop a flow cytometry based immunoassay to measure NER activity in small populations of cells derived from women at risk for or with breast cancer, from ductal lavage or MRI directed biopsy samples of breast epithelial cells or tumor cells, and from appropriate controls. 3) Analyze cell based NER activity from patients samples with clinical, pathological and genetic information, including BRCA1 and BRCA2 carrier status, pathologic grade and stage, familial risk, and molecular correlates					
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Loss of Nucleotide Excision Repair as a Source of Genomic Instability in Breast Cancer

Introduction:

Genomic instability is a hallmark of carcinogenesis in human tumors, including sporadic and familial breast cancer. A major source for genomic instability are defects in mechanisms for the repair of DNA damage or errors in replication. We reported that the breast cancer susceptibility gene BRCA1 is directly involved in the regulation of global genomic nucleotide excision repair (NER) through its ability to transcriptionally regulate DNA damage recognition genes. Several lines of evidence suggest that an altered ability to repair DNA adducts due to cellular defects in NER may be a common source for genomic instability in preneoplastic breast tissue, resulting in enhanced mutagenesis of other cancer genes and breast cancer progression. More recently, we have also discovered that cells missing BRCA1 are deficient in base excision repair (BER) of oxidative DNA damage. Therefore, we are studying NER/BER activity in primary breast epithelial cells and breast cancer tissues from women at risk for or diagnosed with breast cancer, as well as in mammary epithelial cells from mice as a model system. We take advantage of our high-risk breast cancer clinic, where we currently have ongoing clinical protocols using ductal lavage, random periareolar fine needle aspiration (rpFNA) and MRI directed biopsies to screen women for early breast cancer, to study the genetics of breast epithelial cells, and to assess the ability of lovastatin to act as a chemopreventative agent in women carrying BRCA1 mutations. We hypothesize that altered NER/BER activity may be a common, initial and detectable event leading to genomic instability in human breast epithelial cells, ductal carcinoma in situ, or early invasive breast cancers. We are studying NER/BER in preneoplastic breast tissue from high and low risk individuals, BRCA1 mutation carriers and others, and invasive breast cancer tissue, using novel cell based functional assays of DNA repair. Our studies may allow, for the first time, a direct assessment of DNA repair activity in primary breast tissue.

Original Specific Aims and Study Design:

- 1). Apply a recently developed quantitative local DNA damage immunofluorescent assay to measure NER activity in single cells derived from women at risk for or with breast cancer, from ductal lavage, rpFNA or MRI directed biopsy samples of breast epithelial cells or tumor cells, and from appropriate controls.
- 2). Develop a flow cytometry based immunoassay to measure NER activity in small populations of cells derived from women at risk for or with breast cancer, from ductal lavage, rpFNA or MRI directed biopsy samples of breast epithelial cells or tumor cells, and from appropriate controls.
- 3). Analyze cell based NER activity from 25 patient samples per year over 3 years with clinical, pathological and genetic information, including BRCA1 and BRCA2 carrier status, pathologic grade and stage, familial risk, and molecular correlates.

An addition to aim 3, based upon our recent results, is to study BER of oxidative DNA damage (ODD) in mouse mammary epithelial cells from our BRCA1 mouse model, and to assess the level of ODD in primary human mammary epithelial cells obtained by rpFNA from women before and after 6 months of oral lovastatin as part of a chemoprevention trial opened this year by our group.

Identifying altered DNA repair as a central cause of genomic instability relevant to the multistep process of breast cancer carcinogenesis and progression would have major potential impact on clinical risk assessment and both preventative and therapeutic approaches to this disease. A sensitive, quantitative and practical method for assessing functional NER/BER activity in breast epithelial cells may allow for predictive phenotypic risk analysis beyond that currently possible using genotyping approaches. Also, assessment of NER activity of invasive breast cancers using standard diagnostic procedures may allow for selection of individualized therapy based on chemosensitivity.

Research Accomplished According the Statement of Work:

Identifying altered DNA repair as a central cause of genomic instability relevant to the multistep process of breast cancer carcinogenesis and progression would have major potential impact on clinical risk assessment and both preventative and therapeutic approaches to this disease. We have evidence for a novel role for BRCA1 in regulating both NER and BER in human breast epithelial cells. A sensitive, quantitative and practical method for assessing functional NER/BER activity in breast epithelial cells may allow for predictive phenotypic risk analysis beyond that currently possible using genotyping approaches. Also, assessment of NER activity of invasive breast cancers using standard diagnostic procedures may allow for selection of individualized therapy based on chemosensitivity.

A new finding from our laboratory that directly influences this study is that BRCA1^{-/-} murine mammary epithelial cells exhibit increased sensitivity to ODD and the base damaging agent MMS, and decreased BER of ODD compared to wild-type cells. Furthermore, the HMG-CoA reductase inhibitor lovastatin inhibits the increased sensitivity to ODD, specifically in BRCA1^{-/-} cells. Therefore, we have opened a phase II clinical trial of the chemopreventative effects of lovastatin in BRCA1/2 carriers, with cellular atypia of rpFNA derived breast epithelial cell as a primary endpoint, but looking at NER/BER of cells using the techniques developed for this project as a secondary endpoint.

Task 1: Develop quantitative local DNA damage and repair immunofluorescent assay for use on primary human breast tissues.

During the second year of this project we have made considerable progress, and continue to perform a novel technique for the detection of localized DNA damage and damage binding proteins in individual cells, using targeted micro-irradiation techniques and immunofluorescence, based upon recently published work from our laboratory [1, 2]. This has provided a powerful *in vivo* method to analyse the function of proteins that regulate DNA repair (Fig. 1). We adapted this assay to quantitatively measure NER at a single cell level, *in vivo*. Plated cells are UV-irradiated through the 3 μ m isopore filter and either fixed immediately or allowed to repair for 8 or 24 hrs in media and then fixed. Following incubation with monoclonal antibodies to CPDs, as described in Fig. 1, immunofluorescent images of at least 100 cells containing 3 – 7 irradiated sites are captured by a Nikon Eclipse E800 microscope using an RT Slider CCD camera (Spot Diagnostic), and analyzed by Spot RT 3.0 software (Spot Diagnostic) and Quantity One imaging software (Bio-Rad). Spot densitometry is used to analyze average pixel density from all spots detected within at least 100 cells, subtracting background fluorescence from adjacent, non-irradiated nuclear areas.

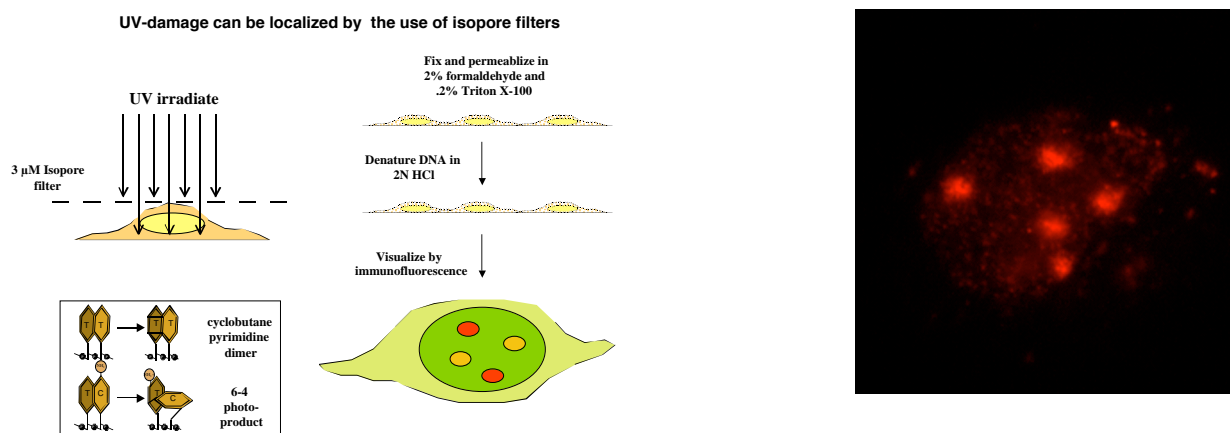


Figure 1. In vivo immunofluorescent detection of localized UV-irradiation induced DNA damage and cellular proteins. Cells are grown on glass coverslips. An isopore filter of 3 μ m size is presoaked in PBS and placed over the cells, and the cells irradiated through the filter with UVC. The cells are fixed with 2% formaldehyde in 0.2% Triton X-100, the DNA denatured by 2N HCl for 5 minutes at 37°, incubated in 20% FBS for 30 minutes to block non-specific binding and incubated sequentially with primary and secondary antibodies. The example pictured at right demonstrates a single cell, with 5 discrete irradiated sites within the nucleus, as detected with monoclonal antibodies to CPDs.

Task 1a: Optimize technique using cell lines in tissue culture.

We have now successfully used this technique to quantitate NER of cyclobutane pyrimidine dimers (CPDs) from wild-type and several NER deficient fibroblast cell lines (XPA, XPC and p53 mutant) and have obtained similar results compared to traditional methods requiring millions of cells and DNA extraction techniques. Therefore, this approach may prove useful for determining NER activity from clinical samples with limited number of cells nor ability to expand in tissue culture. We have also applied this technique to a model cell system. Primary murine mammary epithelial cells were obtained from genetically defined mice allowing for knockout of the mouse Brca1 gene, using a tissue specific conditional cre-lox knockout strategy. We have found that these cells exhibit a significant decrease in NER, as well as increased sensitivity to UV-irradiation. These results are important in that they confirm the utility of this assay in cells relevant to the current study, being breast epithelial cells containing genetic alterations in the BRCA1 gene [3].

Task 1b: Optimize technique for use with human breast epithelial cells.

During year two, we collected 30 new samples of human primary breast epithelial cells by rpFNA. In general, this procedure provided for far better cell yields than the originally proposed ductal lavage technique. The majority of these samples had sufficient cell numbers to submit for cytologic assessment of atypia, immunohistochemistry for biomarkers, and for laboratory studies of both NER (as in Figure 1) and BER (described below).

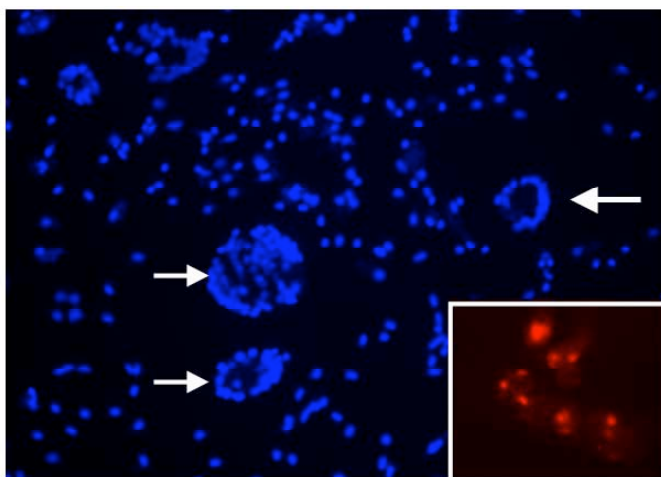


Figure 2. In vivo immunofluorescence and detection of localized DNA damage in primary breast epithelial cells collected by ductal lavage. Breast epithelial cells were collected as detailed in the appended clinical protocol, and processed as described in Fig. 1. The image demonstrates ductal architecture of these samples, and the insert shows detection of localized DNA damage using techniques as described above.

Develop Comet assay to measure ODD in cultured cell.

In laboratory studies, we recently found that BRCA1 deficient murine mammary epithelial cells were 10-fold more sensitive to hydrogen peroxide induced cytotoxicity than their wild-type counterparts. Furthermore, treatment with non-cytotoxic (1 μ M) doses of the HMG-CoA reductase inhibitor lovastatin inhibited the enhanced cytotoxicity and oxidative DNA damage induced in BRCA1 deficient cells (Figure 3).

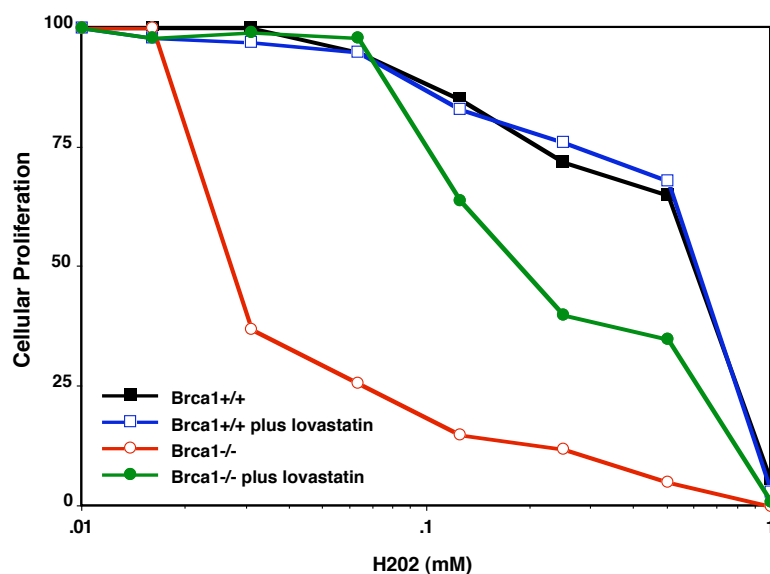


Figure 3: Inhibition of oxidative DNA damage induced cytotoxicity in BRCA1^{-/-} cells by lovastatin. BRCA1 wild-type (+/+) and null (-/-) murine mammary epithelial cells derived from transgenic mice were incubated for 24 hours in the presence or absence of 1 μ M lovastatin, and then treated with varying concentrations of hydrogen peroxide (H2O2) for a further 72 hours, in 96 well tissue culture plates. MTT assays were performed to determine cellular proliferation, and graphed as the percentage of cell growth compared to untreated controls.

To specifically analyze the role of BRCA1 and lovastatin on oxidative DNA damage we used the “alkaline Comet” or single cell gel electrophoresis assay, combined with the lesion specific DNA glycosylase enzyme FPG (formamidopyrimidine [fapy]-DNA glycosylase) for measuring oxidative DNA damage and repair. Briefly, following treatment with H2O2 \pm lovastatin, cells are embedded in low melting point agarose on two precoated standard microscope slides. Cells

are lysed with an alkaline buffer, one slide is treated with the FPG enzyme, and the second slide is used as a control. Electrophoresis is performed under denaturing conditions and cells and comet tails visualized by staining the nucleic acid with SYBR green fluorescent dye. ODD levels are determined using fluorescent microscopy and degree of DNA damage assessed by measuring the comet tail moment, Olive moment, and percent DNA in the comet tail. BRCA1 mutant cells exhibit increased oxidative DNA damage, and this damage is strongly inhibited in the presence of lovastatin (Figure 4). Furthermore, this effect appears specific to BRCA1^{-/-} cells.

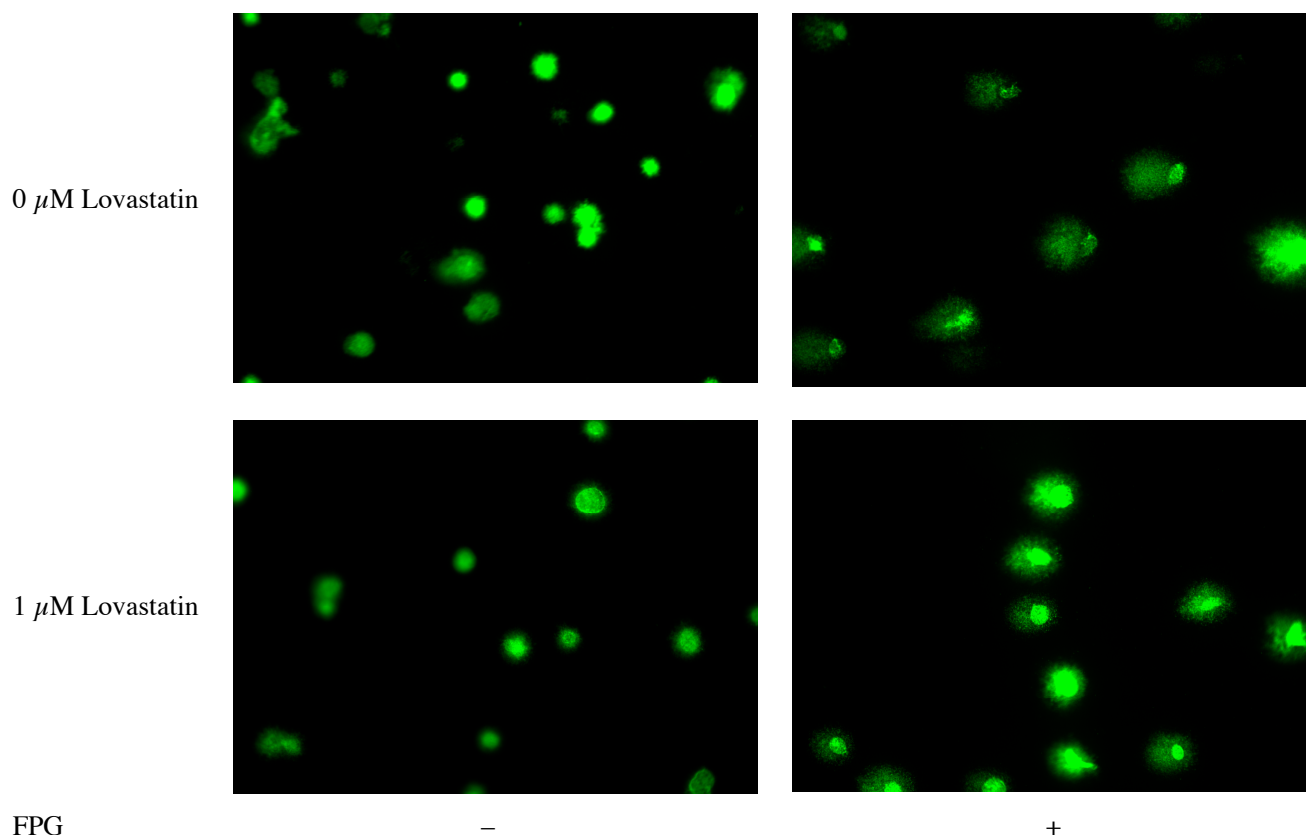
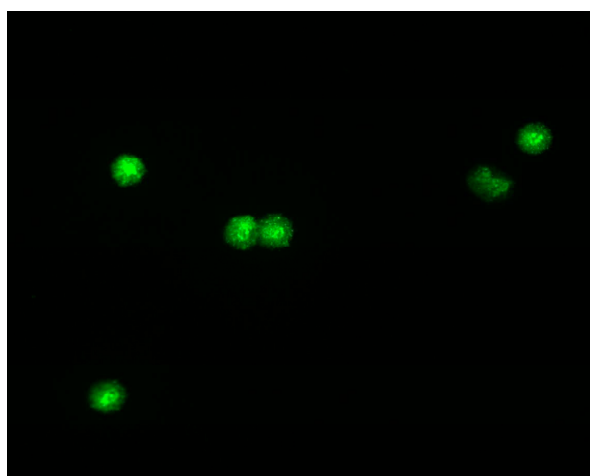


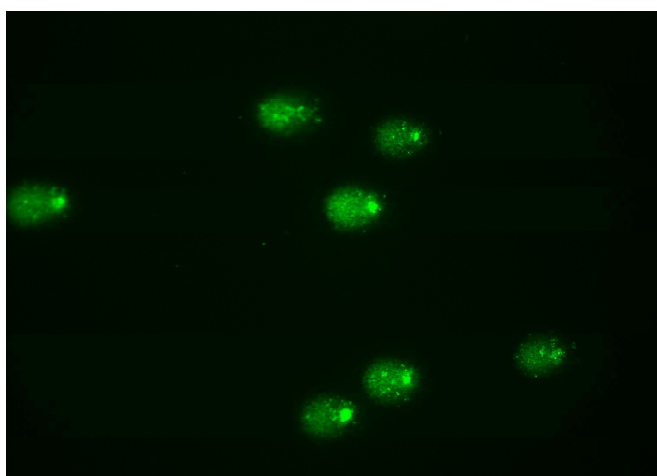
Figure 4: Inhibition of oxidative DNA damage induced strand breaks in BRCA1^{-/-} cells by lovastatin. BRCA1 null (^{-/-}) murine mammary epithelial cells derived from transgenic mice were incubated for 24 hours in the presence or absence of 1 μ M lovastatin, and then treated with hydrogen peroxide (H₂O₂) and then embedded in low melting point agarose on precoated microscope slides. Cells were lysed with an alkaline buffer and treated with the FPG enzyme to nick DNA at sites of 8-oxoguanine base lesions. Electrophoresis was performed under denaturing conditions and cells and comet tails visualized by staining the nucleic acid with SYBR green fluorescent dye. As can be seen, the FPG induced comet tails (reflecting a higher level of oxidative base damage) in BRCA1^{-/-} cells are significantly reduced in length by the presence of lovastatin.

Optimize Comet assay for use with human breast epithelial cells.

Based upon our laboratory studies demonstrating that BRCA1 knockout murine mammary epithelial cells sustain elevated ODD associated with defective base excision DNA repair, we have evaluated ODD in clinical breast epithelial cells samples from women carrying BRCA1 mutations. We have now optimized the Comet assay for use with clinical samples, and developed appropriate controls (MCF-7 breast cancer cells treated with ionizing radiation) to run in parallel with each clinical specimen. Cells from the rpFNA specimen are embedded in low melting point agarose and the full specimen is split between two precoated standard microscope slides. Cells are lysed with an alkaline buffer, one slide is treated with the FPG enzyme, and the second slide is used as a control. Electrophoresis is performed under denaturing conditions and cells and comet tails visualized by staining the nucleic acid with SYBR green fluorescent dye. ODD levels are determined using fluorescent microscopy and degree of DNA damage assessed by measuring the comet tail moment, Olive moment, and percent DNA in the comet tail. Our initial results are remarkable. In the first six clinical samples, we have seen a complete concordance between cellular atypia and Comet assay DNA tails suggesting significant ODD (Figure 5). No patient with normal breast duct cells has been found to have ODD.



Minus FPG



Plus FPG

Figure 5: Oxidative DNA damage in primary human breast epithelial cells. Breast epithelial cells from a BRCA1 mutation carrier were acquired by rpFNA and then embedded in low melting point agarose on precoated microscope slides. Cells were lysed with an alkaline buffer and treated with the FPG enzyme to nick DNA at sites of 8-oxoguanine base lesions. Electrophoresis was performed under denaturing conditions and cells and comet tails visualized by staining the nucleic acid with SYBR green fluorescent dye. Cytology of these same cells confirmed atypia.

Task 2. Develop flow cytometry based immunoassay for measurement of NER in primary human breast tissues.

During year two, we did not further develop this aim, but rather focused our efforts on developing the Comet assay for measurement of ODD in cell lines and clinical specimens. However, we plan to further pursue this aim during year three, using both antibodies to cyclobutane pyrimidine dimers, as initially proposed, as well as to 8-oxoguanine, for measurement of ODD, using flow cytometry.

Task 3 and 4. Collect primary breast epithelial cells from women undergoing comprehensive screening using ductal lavage, rpFNA and MRI directed biopsies.

During year two, we collected 30 clinical samples of breast epithelial cells from women with a high risk for hereditary breast cancer on one of our several IRB approved clinical protocols, for analysis of NER and BER. Because of the results with BRCA1-/- cells on reversal of ODD by lovastatin, we opened a new phase II trial for chemoprevention with lovastatin in women BRCA carriers (consent attached in appendix). This serves as an additional source for specimens for the current study, as all women are also consented for analysis of breast epithelial cells for DNA repair activity. Cell yield from rpFNA has been substantially better than was the case with ductal lavage, and samples are adequate for cytologic examination and both NER and BER assays. Having optimized the functional NER assay for use with clinical samples, and developed the Comet assay for assessing ODD in samples, during year three we hope to begin correlating functional DNA repair phenotypes with genotypes and clinical outcomes. We have already seen a tight correlation between cytologic atypia and high levels of ODD in clinical specimens, and will follow this up with repeat rpFNAs in the same patients following 6 months of lovastatin treatment.

Key Research Accomplishments:

- Development and use of a quantitative immunoassay for measuring NER at a single cell level in cell lines and clinical specimens.
- Development of Comet assay to demonstrate decreased BER in Brca1-/- murine mammary epithelial cells.
- Use of Comet assay to detect ODD in clinical specimens.
- Demonstration of feasibility of collection and laboratory procedure for introducing and detecting DNA damage in clinical samples.
- Use of rpFNA as a more reliable and robust method for collection of human breast ductal epithelial cells from 30 patients.

Reportable Outcomes:Publications:

Kurian AW, Mills MM, Jaffee M, Sigal BM, Chun NM, Kingham KE, Collins LC, Nowels KW, Plevritis SK, Garber JE, Ford JM and AR Hartman. Ductal lavage of fluid-yielding and non-fluid-yielding ducts in BRCA1 and BRCA2 mutation carriers and other women at high inherited breast cancer risk. *Cancer Epidemiol Biomarkers Prev* **14**: 1082 – 1089 (2005).

Kurian AW, Hartman AR, Mills MA, Ford JM, Daniel BL, and Plevritis SK. Opinions of women with high inherited breast cancer risk about prophylactic mastectomy: an initial evaluation from a screening trial including magnetic resonance imaging and ductal lavage. *Health Expectations* **8**: 221 - 233 (2005).

Ford JM. Regulation of DNA damage recognition and nucleotide excision repair: another role for p53. *Mutation Research* **577**: 195-202 (2005).

Daly MB, Axilbund JE, Bryant E, Buys S, Eng C, Friedman S, Esserman LJ, Farrell CD, Ford JM, Garber JE, Jeter JM, Kohlmann W, Lynch PM, Marcom PK, Nabell LM, Offit K, Osarogiagbon RU, Pasche B, Reiser G, Sutphen R and Weitzel JN. Genetic/Familial high-risk assessment: breast and ovarian. *J Natl Compr Canc Netw*. **4**: 156 – 176 (2006).

Ghanouni P, Kurian AW, Margolis D, Hartman AR, Mills MA, Plevritis SK, Ford JM and Daniel BL. Ductal pattern enhancement on magnetic resonance imaging of the breast due to ductal lavage. Submitted, *Breast J*. (2006).

Sharma VB, Hartman AR, Sgasias MK, Turner S, Cowan K and Ford JM. Loss of nucleotide excision repair in brca1-deficient murine mammary epithelial cells. *Submitted, Cancer Research, in revision* (2006).

Abstracts:

Lin PS, Sage J, Ford JM. The role of the Rb/E2F tumor suppressor pathway in DNA damage repair. *Proc. Am. Assoc. Cancer Res.* **46**: 1623 (2005).

Sharma VB, Hartman AR, Cowan K, Ford JM. Enhanced sensitivity to cisplatin and gemcitabine in DNA repair deficient Brca1 null mouse embryonic fibroblasts. *Proc. Am. Assoc. Cancer Res.* **46**: 4390 (2005).

Presentations:

Role of p53 and BRCA1 in DNA Repair. The Cancer Institute of New Jersey Grand Rounds Speaker. New Brunswick, NJ. April 6, 2005.

Regulation of Human Nucleotide Excision Repair by Tumor Suppressor Genes. Invited Speaker for session on "Models and Mechanisms for Processing DNA Damage." 9th International Conference on Environmental Mutagens / 36th Annual Meeting of the Environmental Mutagen Society. San Francisco, September 8, 2005.

Advances in Diagnosis and Treatment of Breast Cancer. Makati Medical Center, Manila, Philippines, Sept. 26 & 27, 2005. Hereditary Breast Cancer Syndromes

2nd Global Medical Forum Symposium: Advances in Genetic Aspects, Diagnosis and Treatment of Breast Cancer. Jaslok Hospital and Hinduja Hospital, Mumbai, India, October 1 & 2, 2005.

Genetic Testing for Hereditary Cancer Syndromes: Who, What, When, Where and Why. San Francisco Surgical Society, San Francisco, CA, January 18, 2006.

Assessing Hereditary Cancer Risk: Appropriate Use of Cancer Genetic Testing and Clinical Management. Palo Alto Medical Foundation, January 23, 2006.

Conclusions:

The overall goal of this project is to determine if DNA repair activity can be reliably and practically quantitated from breast epithelial tissues and breast cancer cells derived from women undergoing standard screening and diagnostic procedures. We have developed and validated laboratory assays allowing for this question to be tested in a clinical testing, and have organized a clinical trial to facilitate collection of tissues and outcomes information. Should a DNA repair defect appear to be present in a subset of these women, we will proceed to test our hypothesis in a prospective trial to determine the role of NER/BER in breast cancer risk and clinical outcomes. Furthermore, we are prospectively testing whether a chemopreventative agent may reduce the amount of DNA damage that occurs in breast epithelial cells, using these same techniques. Therefore, the results of our current proposed study could have a major impact on risk assessment and prevention of breast cancer. In addition, since NER is a key pathway affecting the sensitivity of tumor cells to several types of cancer chemotherapeutic drugs, our results could have implications for tailoring drug treatment in invasive breast cancer.

References:

1. Fitch ME, Cross I and Ford JM. p53 responsive nucleotide excision repair gene products p48 and XPC, but not p53, localize to sites of UV-irradiation induced DNA damage. *Carcinogenesis* **24**: 843 – 850 (2003).
2. Fitch ME, Nakajima S, Yasui A and Ford JM. *In vivo* stimulation of XPC protein binding to UV-induced cyclobutane pyrimidine dimers by the DDB2 gene product. *J. Biol. Chem.*, **278**: 46906 – 46910 (2003).
3. Sharma VB, Hartman AR, Cowan K, Ford JM. Enhanced sensitivity to cisplatin and gemcitabine in DNA repair deficient Brca1 null mouse embryonic fibroblasts. *Proc. Am. Assoc. Cancer Res.* **46**: 4390 (2005).

Appendices:

1. Stanford IRB Approved Consent Form for trial “Chemoprevention of Breast Cancer with Lovastatin.”

Study Title: A Phase II Trial of Lovastatin for Modification of Abnormal Breast Duct Cytology and Risk-Associated Biomarkers in Women at High Inherited Risk of Breast Cancer

INFORMED CONSENT FORM

Are you participating in any other research studies? _____ Yes _____ No

INTRODUCTION TO RESEARCH STUDIES

A research study is designed to answer specific questions, sometimes about a drug or device's safety and its effectiveness. Being in a research study is different from being a patient. When you are a patient, you and your personal doctor have a great deal of freedom in making decisions about your health care. When you are a research subject, the Protocol Director and the research staff will follow the rules of the research study (protocol) as closely as possible, without compromising your health.

PURPOSE OF RESEARCH

You are being asked to take part in this research study because you have a strong family history of breast or ovarian cancer, or you have been found to have an increased chance of developing inherited breast cancer. You are being asked to take part because your risk of developing breast cancer is much higher than most women's risk. You are being asked to take part because you have heard about, but for now have not chosen to take, other steps which could reduce your breast cancer risk, such as prophylactic mastectomy (surgery to remove normal breast tissue in the hope of preventing breast cancer) or taking a drug called tamoxifen.

The purpose of this research study is to find out what effects (good and bad) Lovastatin has on you and your risk of developing breast cancer. Lovastatin is a drug that is approved by the Food and Drug Administration for the treatment of high cholesterol, and it has been widely used and found to be safe in patients who take it for treatment of high cholesterol. However, we do not know whether it can have an effect on the risk of breast cancer. We will determine this by examining cells from your breasts (which we will collect using a small needle) for abnormalities, which are common among women like you and may predict increased breast cancer risk, before and after you take Lovastatin for six months. If Lovastatin can decrease these abnormalities, then it might be able to decrease your risk of breast cancer.

This research is being done because currently, there is no method to reduce the risk of breast cancer which is proven to be both effective and acceptable to most women like you. The goal of this study is to find out whether Lovastatin can decrease breast cancer risk, and whether it will be an easy drug to tolerate (whether it will have few bad effects, and whether you will find it acceptable to take every day).

Your participation in this study is entirely voluntary.

Your decision whether or not to participate will not prejudice you or your medical care. If you wish to participate in this study, you must sign this form. If you decide to participate, you are **free to withdraw** your consent, including your authorization regarding the use and disclosure of your health information, and to discontinue participation at any time without prejudice to you or effect on your medical care. If you decide to terminate your participation in this study, you should notify Dr. Allison Kurian and the

Study Title: A Phase II Trial of Lovastatin for Modification of Abnormal Breast Duct Cytology and Risk-Associated Biomarkers in Women at High Inherited Risk of Breast Cancer

study coordinator, Meredith Mills, at 650-724-5223.

This research study is looking for 88 women with a high inherited risk of breast cancer.

DURATION OF STUDY INVOLVEMENT

We think you will be in the study for six months: this will be the time when you are being seen by the doctor every month, and when you are taking the study drug, Lovastatin. After the study has concluded, we may contact you to ask about your current health status (this could occur a year or more after the study has finished), or about future studies that might be of interest to you.

PROCEDURES

The schedule of this study is shown in the table below:

Study Test	Study Entry (before Month 1)	Month 1	Month 2	Month 3	Month 4	Month 5	Study End (after Month 6)
Visit to doctor's office for physical exam	X						X
Questions asked by doctor and other study staff about your health and symptoms	X			X			X
Blood test to monitor for drug side effects (to liver and muscle) Pregnancy Test (study entry only)	X			X			X
Blood test to monitor drug effect on cholesterol and research sample	X						X
Breast needle aspiration	X						X
Mammogram	X						X
Breast magnetic	X						X

Study Title: A Phase II Trial of Lovastatin for Modification of Abnormal Breast Duct Cytology and Risk-Associated Biomarkers in Women at High Inherited Risk of Breast Cancer

resonance imaging (MRI) (Optional)							
Urine Sample	X						X

If you take part in this study, you will have the following tests and procedures performed by Dr. James Ford, Dr. Allison Kurian, or their research staff:

1) Procedures that are part of breast cancer screening or standard medical care for a woman like you, and may be done even if you do not join the study:

A) Visit to doctor for physical examination, including breast examination: done twice, at the beginning of the study and 6 months later, at the end. A physical examination involves the doctor talking with you to find out whether you have had any symptoms or problems, and then looking in your eyes and mouth, examining your neck, underarm, and breast areas, listening to your heart and lung, examining your abdomen, and your arms and legs.

B) Mammogram: done twice, at the beginning of the study and 6 months later, at the end. You will visit the hospital for this test, but will not need to stay overnight. A mammogram involves placing your breast in a machine, which will squeeze it between two plates to make the breast easier to see on the X-ray images. Most women do not find this painful, though some may find it uncomfortable. Approximately two X-ray pictures will be taken of each breast, but if the technician sees an abnormality, he or she may recommend that more images be done. The process generally takes about 30 minutes.

C) Breast magnetic resonance imaging (MRI): may be done twice, at the beginning of the study and 6 months later, at the end. Here is a description what you will experience when you have a breast MRI:

The breast MRI machine uses a strong magnet and radiofrequency magnetic fields to make images of the body interior. The scanning procedure is very much like an X-ray CT scan. You will be asked to lie on your stomach on a long narrow couch in the center of a large magnet in the shape of a tube, while the machine gathers data. The padded table will have two shallow cups into which you will place your breasts. The tube is well lit and has a speaker system so that you can talk to the operator of the machine. On the day of the study, you will first undergo MRI without injection of contrast agent: contrast agent is a liquid that is inserted into the vein, and helps to make any abnormal area of the breast more visible. You will then receive an intravenous catheter or “IV” in a vein of your arm, for the purpose of contrast injection. This MRI will take 15-30 minutes. Contrast agent will then be injected in your vein and you will have another MR scan immediately following this injection. After the examination is completed, the catheter will be removed from your vein. This MRI will take 15-30 minutes. During this time you will not be exposed to x-rays, but to a strong magnetic field and radiofrequency magnetic fields. You will not feel either. You will, however, hear repetitive tapping noises that arise from the MRI scanner. We will provide earplugs or headphones that you will be required to wear. The space within the large magnet in which you lie is somewhat confined, although we have taken many steps to relieve the claustrophobic or “closed-in” feeling. Please take note that

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some people have experienced claustrophobia, or a closed-in feeling, which required treatment with a mild sedative while in the MRI scanner; a mild oral sedative drug, lorazepam, will be offered to you if you have had difficulty with enclosed spaces in the past. Most women who have undergone this examination have not required the sedative medication. If you do wish to take this medication, you should take it about one hour before the MRI, and you should not drive a car home afterwards, but should arrange for someone to give you a ride.

You can discontinue the exam at any time. You will visit the hospital for this test, but will not need to stay overnight. The breast MRI machine can examine only one breast at a time, so it will be necessary for you to return to the hospital 1-3 days later to have an MRI of the other breast. For the second MRI, you will require placement of a second catheter in your vein for intravenous contrast, which will be removed after the examination.

D) Blood draw to test your cholesterol levels, to see whether the study drug, Lovastatin, has changed them: done twice, at the beginning of the study and 6 months later, at the end, when you visit the doctor's office. One tube of blood containing 5 to 10 milliliters (about 1 to 2 teaspoons full) will be taken for this test. The blood sample will be destroyed after it has been drawn and analyzed. An additional sample will be taken at the same time for research purposes. This sample will be stored in our laboratory with a unique identifier.

E) Blood draw to test your blood counts and electrolytes and make sure they are normal prior to study entry and after the study ends: done twice, at the beginning of the study and 6 months later, at the end, when you visit the doctor's office. Two tubes of blood, containing a total of 10-20 milliliters (about 2 to 4 teaspoons full) will be taken for this test. The blood sample will be destroyed after it has been drawn and analyzed.

2) Standard procedures being done because you are in this study:

A) Blood draw to monitor liver and muscle function and make sure there is no damage from the study drug, Lovastatin: done when you visit the doctor's office at the beginning of the study, your visit in the third month of the study, and on your visit at the end of the study (a total of 3 times). One tube of blood containing 5 to 10 milliliters (about 1 to 2 teaspoons full) will be taken for this test. The blood sample will be destroyed after it has been drawn and analyzed.

B) Random periareolar fine needle aspiration (rpFNA): a small needle will be used to remove a few cells from two areas of each breast that have no masses or abnormalities on mammogram, MRI or ultrasound – parts of the breast that are seen to be normal – in order to look for abnormalities. This procedure is very commonly done to diagnose breast cancer. In this study, it will be done to look for early changes in your breast cells which might develop into breast cancer. Here is a description of what you will experience when you have an rpFNA:

Three weeks before you have the rpFNA, you will be asked not to take aspirin, ibuprofen, motrin, naproxen, celecoxib, or other drugs known as non-steroidal anti-inflammatory agents (NSAIDs). This

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is to reduce the risk of any bleeding or bruising at the time of the rpFNA, which can happen more often when patients take NSAIDs. However, taking Tylenol (acetaminophen) is acceptable, because it will not cause increased bleeding. On the day of the rpFNA procedure, a mild oral sedative, lorazepam, will be prescribed for you if you wish; this is a medication which decreases anxiety, and may cause you to feel drowsy. Most women who have undergone this procedure have not required the sedative medication. If you do wish to take this medication, you should take it about one hour before the rpFNA procedure, and you should not drive a car home afterwards, but should arrange for someone to give you a ride.

The procedure will begin with cleansing two areas close to the nipple on each breast with alcohol. Next these areas will be injected with a numbing medication, lidocaine, so that you will not have pain with the rpFNA procedure. Please tell the investigators before the procedure if you have ever had an allergic reaction to lidocaine or another anesthetic drug. A small needle (the same size as the needles which are routinely used to draw blood) attached to a syringe will then be inserted into one area next to your nipple which has been numbed; if this is painful, you should tell the investigator performing the procedure, who can give you more numbing medication. A small sample of fluid and tissue will be drawn into the syringe, and will then be sent to the laboratory for evaluation. After that, this process will be repeated on the other side of your nipple, in an area which has also been numbed. After that, the procedure will be repeated in two areas near the nipple of your other breast. Once the procedure is finished, ice packs will be placed over the areas where the needle has been inserted into your breasts for 10 minutes, and then your breasts will be bandaged in gauze, which will also be wrapped around your chest. You will be asked to wear a tight-fitting athletic bra home from the clinic, and for the next 4-5 days; this will apply pressure and decrease the chance of any bruising. The whole procedure will take approximately 30 minutes.

Most women who have had this procedure find that it is not very uncomfortable (a 2 on a scale of 1-10, with 1 being almost no pain, and 10 being the most severe pain), and have not had significant pain or bruising after the procedure. However, if you do have mild pain, you should not take aspirin, ibuprofen, motrin, naproxen, celecoxib, rofecoxib or other drugs known as non-steroidal anti-inflammatory agents (NSAIDs) for the next 3 weeks, because they increase the risk of bruising or bleeding. You may take acetaminophen (Tylenol). If the pain is severe or lasting, or if there is lasting bleeding, bruising, or any other problem, please call the study investigators right away.

The rpFNA procedure will be done two times, 6 months apart: once before you start the study drug, Lovastatin, and once afterwards, to see whether Lovastatin has caused a change in the appearance of your breast cells. It will be done when you visit the doctor's office, and you will not need to go to the hospital for this or to stay overnight.

If you take part in this study, **you will be asked not to take certain other medications.** You will be asked not to take any NSAIDs more than two times weekly while you are in the study, because these drugs are also under study for their effect on the breast changes we plan to measure in you. We ask you not to take them because in this study, we want to discover what effect Lovastatin has without the presence of other drugs such as NSAIDs.

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The other drugs we ask you to take no more than two times weekly include ranitidine (zantac), famotidine (pepcid), nizatidine (axid), omeprazole (prilosec), esomeprazole (nexium), or lansoprazole (prevacid). These are drugs which treat acid reflux or heartburn, and they may interfere with the effects of the study drug, Lovastatin, if taken often. You may take drugs like Maalox or Mylanta for this condition if needed. We also ask you not to drink more than three alcoholic drinks per week while you are in the study because alcohol can increase your risk of liver side effects. Also we ask that you do not take any vitamins at a strength greater than a multivitamin.

We ask you not to drink one quart or more of grapefruit juice per day, as this has been shown to cause liver problems when people are also taking lovastatin. Drinking less than this amount (for example, one or two glasses per day) is safe.

We ask that you call the study investigators before starting to take any new drug, vitamin, or supplement while you are participating in the study, whether or not it is a drug that requires a prescription from a doctor.

C) Blood draw at the beginning and end of study (when you have finished the drug) to be used for research purposes only.

D) A urine sample will be collected at the beginning of the study and at the end of the study (when you have finished taking all of the lovastatin). This will be used for research purposes only.

<h3>TISSUE SAMPLING FOR RESEARCH TESTING</h3>
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Research using tissues is an important way to try to understand human disease. You have been given this consent form because the investigators want to include your breast cells in a research project. Before allowing your breast tissue to be studied, you should know that you will be told the results of all tests in this study which may affect your health. These include mammogram, breast MRI, all blood tests, and the results of the analysis of your breast cells on rpFNA.

After analysis of your breast tissue to see whether the cells appear normal or abnormal, the sample of your breast tissue will forever be separated or unlinked from your name. This will protect your identity and preserve anonymity. Once you donate the sample, you will not be able to withdraw your tissues from the research project because the samples will not be traceable. Other research tests will be performed on these tissues to help the researchers learn more about your condition, but these will not be tests with results that will affect your health. For that reason, you will not be informed of the results of these tests. These tests may include analysis of DNA (the nucleic acids contained in your cells that encode your genetic “blueprint”) for genetic mutations, analysis of RNA expression levels, analysis of protein levels of important gene products involved in cancer, and development of tissue culture cell lines. If any of these studies result in a therapeutic treatment option, then a separate detailed consent form outlining this treatment will be given to you. The tissues removed from you may be useful for research, development, or education, resulting in new drugs, therapies, diagnostic procedures or the like becoming available to the public. Although these uses will not benefit you directly, the investigators ask

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that you consent to these uses and donate such tissues by signing this form. The investigator will use the results of this study as research only, and not include them in your medical record. Generally, you will not be told the results, even if their might be some potential benefit for you.

Disease testing and genetic research raise certain questions about informing you of any results. Possible risks of knowing results include anxiety, other psychological distress, and the possibility of insurance and job discrimination. A possible risk of not knowing includes being unaware of the need for treatment. These risks can change depending on the results of the research, and whether there is a treatment or cure for a particular disease. Donation of tissue for these research purposes is not genetic testing.

☐ **I do** ☐ **I do not** consent to donate my blood serum sample, a portion of my breast tissue sample and my urine sample for use in breast cancer research.
(initial the appropriate box above)

SUBJECT'S RESPONSIBILITIES

If you choose to participate, you should:

- 1) Take the study drug as instructed
- 2) Keep your study appointments. If it is necessary to miss an appointment, please contact the investigators or program coordinator to reschedule as soon as you know you will miss the appointment.
- 3) Tell the investigators or program coordinator about any side effects, doctor visits, or hospitalizations that you may have.
- 4) Tell the investigators or program coordinator if you believe you might be pregnant.
- 5) Keep the study drug in a safe place, away from children and for your use only.
- 6) Keep your medication diaries as instructed.
- 7) Ask questions as you think of them.
- 8) Tell the investigators or program coordinator if you change your mind about staying in the study.
- 9) You will have to sign this consent and Authorization form if you want to participate in this research study.

While participating in this research study, you should not take part in any other research project without approval from the study investigators. This is to protect you from possible injury arising from such things as extra blood drawing, extra x-rays, the possible interaction(s) of research drugs, or other similar hazards.

WITHDRAWAL FROM STUDY

If you first agree to participate and then you change your mind, you are **free to withdraw** your consent and discontinue your participation at any time. Your decision will not affect your ability to receive

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medical care for your disease and you will not lose any benefits to which you would otherwise be entitled. There are no anticipated consequences to withdrawal from the research study.

If you withdraw from the study, or the study medication is stopped for any reason, please notify the investigators, Dr. James Ford, Dr. Allison Kurian, or the study coordinator, Meredith Mills, at 650-724-5223. You will be asked to return all of the study medication, Lovastatin, if you choose to withdraw.

The investigators may decide to take you off this study if they think that it is in your best medical interest, if funding for the study is stopped, if you develop a change in your health which could make participating dangerous for you, if new information about this drug, or your breast cancer risk and its management, becomes available, or for other unanticipated circumstances.

POSSIBLE RISKS, DISCOMFORTS, AND INCONVENIENCES

While on the study, you are at risk for some known side effects. You should discuss these with the researcher and/or your regular doctor. There also may be other side effects that we cannot predict. Other agents may be given to make any side effects which happen less serious and uncomfortable. Many side effects go away shortly after you stop participating in the study, but in some cases side effects can be serious or long-lasting or permanent.

Risks and side effects related to participating in this study include:

- 1) **Side effects which have been seen in people taking Lovastatin**, the study drug:

Side effect	Likelihood and Importance	Duration
Rise in blood levels of liver enzymes, because of minor liver damage	Unlikely; serious in some cases, not serious in most	Usually goes away after stopping Lovastatin
More serious liver damage	Very unlikely, but serious	May go away after stopping Lovastatin, but in some cases may be permanent
Muscle damage, with pain and rise in blood level of muscle enzymes	Unlikely; may be serious	Usually goes away after stopping Lovastatin
More serious muscle damage, which could affect kidney function	Very unlikely, but serious	May go away after stopping Lovastatin, but in some cases may be permanent
Joint aches	Unlikely, not serious	Usually goes away after stopping Lovastatin
Sinus discomfort	Unlikely, not serious	Usually goes away after stopping Lovastatin

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Dry cough	Unlikely, not serious	Usually goes away after stopping Lovastatin
Stomach discomfort or nausea	Unlikely, not serious	Usually goes away after stopping Lovastatin
Loose bowel movements	Unlikely, not serious	Usually goes away after stopping Lovastatin
Difficulty sleeping	Unlikely, not serious	Usually goes away after stopping Lovastatin
Headache or fatigue	Unlikely, not serious	Usually goes away after stopping Lovastatin
Discomfort with urinating or bladder infection	Unlikely, not serious	Usually goes away after stopping Lovastatin

Except for the rare cases of liver or muscle damage, the rest of these side effects are about as common in people taking Lovastatin as in people who are not taking any drug.

2) Risks related to having a breast needle aspiration

The risks of having a breast needle aspiration are similar to the risks of having a blood draw: there is a low risk of bleeding and a low risk of infection any time the skin is pierced by a needle. Eight out of 10 women find that a breast needle aspiration is very tolerable, with little pain or other difficulty. About 1 in 100 women will have severe bruising, which might need further treatment; this is much less likely when women do not take NSAIDs, including aspirin, ibuprofen, motrin, naproxen, celecoxib, rofecoxib for three weeks before the rpFNA procedure.

3) Risks of having a mammogram:

The risks of having a mammogram include discomfort from having your breast squeezed by the mammogram machine, which is temporary. There is some exposure to radiation when you have a mammogram, but it has been determined that the amount of radiation which you receive is very low, and very unlikely to cause any harm.

4) Risks of having a breast MRI:

Magnetic fields do not cause harmful effects at the levels used in the MRI machine. However, the MR machine uses a very strong magnet that attracts some metals, and can affect some electronic devices. If you have a cardiac pacemaker or any other medical device in or on your body, it is very important that you tell the MRI operator and study investigators immediately. Metal objects may be strongly attracted to the magnet, so it is very important that you tell the MRI operator about any metal objects, including surgical clips, devices or implants that are in or on your body before entering the magnet room. In some cases, if you have those devices you should not have an MRI examination. Watches and credit cards should also be removed, because these items could be damaged by the MRI magnet; you will be provided a way to secure these items. If you have ever had a head or eye injury that involved metal fragments, if you have ever worked in a metal shop, or if you could be pregnant, you should tell the MRI operator or the study investigators **before having an MRI**.

There is a possibility that you could experience a twitching feeling because of the magnetic field changes during the scan; this is expected and should not be painful. If you have had severe allergies, or ever had

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an allergic reaction to contrast agents for imaging, especially those using gadolinium, please tell the MRI operator or the study investigators before having an MRI. **If you feel discomfort at any time, you should tell the MRI operator, who will stop the exam at any time you wish.**

5) Risks of having a blood draw:

The risks of having a blood draw include bleeding, bruising and infection, which are risks whenever the skin is pierced by a needle. These risks are very unlikely to be serious.

6) Reproductive risks:

If you are able to become pregnant, and choose to participate in this study, it is expected that you will use an effective method of birth control to prevent exposing a fetus to Lovastatin, which may be dangerous to a developing fetus. If you are pregnant or currently breast feeding, you may not participate in this study. If you are pregnant, if you become pregnant, or if you are breast-feeding during this study, you or your child may be exposed to risk of birth defects if you are taking Lovastatin.

If you choose to participate in this study:

- 1) To confirm to the extent possible that you are not pregnant, you must agree to have a pregnancy test done before beginning this research study.
- 2) You must agree to avoid sexual intercourse or use an effective birth control method, including oral contraceptive pills, intrauterine device (IUD), or barrier method (including diaphragm or condoms used as recommended).
- 3) You must accept the risk that pregnancy could still result despite the responsible use of a reliable method of birth control.
- 4) You must agree to notify the investigator as soon as possible of any failure of proper use of your birth control method, or if you become pregnant, either of which may result in your being withdrawn from the study.

7) Risks related to developing breast cancer

This is a study of Lovastatin for its potential to decrease your risk of breast cancer. However, we are doing the study because we do not know whether it will work, and you will continue to remain at high risk of breast cancer while you are participating in this study. There are other options to participating in the study, which might reduce your risk of breast cancer. **Please review these options**, which are listed in the section, “**ALTERNATIVES**” below.

For more information about risks and side effects, ask the researchers, Dr. Allison Kurian and Dr. James Ford, or contact Meredith Mills, the study coordinator, at 650-724-5223.

POTENTIAL BENEFITS

This is a study to determine whether Lovastatin can help to reduce your risk of breast cancer. Therefore, there is a possibility that you may benefit by having a decreased risk of breast cancer after participating in this study. We hope the information learned from this study will benefit other patients at high risk of breast cancer in the future.

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The study is being performed because we do not know whether Lovastatin will help to reduce your breast cancer risk. **Therefore, we cannot and do not guarantee or promise that you will receive any benefits from this study.**

ALTERNATIVES

Instead of being in this study, you have these options:

- 1) **Reduce your risk of developing breast cancer by having a prophylactic mastectomy** (surgery to remove both breasts to prevent a cancer), or a prophylactic bilateral salpingo-oophorectomy (surgery to remove both ovaries, which leads to a reduced risk of breast cancer if you have not yet undergone menopause).
- 2) **Reduce your risk of breast cancer by screening:** this involves undergoing breast examinations twice yearly by your physician, performing breast self examination monthly, and having either yearly mammography alone, or yearly mammography and breast magnetic resonance imaging (MRI).
- 3) **Take tamoxifen**, a daily pill which in some women may reduce the risk of developing breast cancer. Side effects include hot flashes (common), risk of blood clots and cancer of the uterus (rare but can be serious or fatal).
- 4) **Decide not to take any steps to reduce your risk of breast cancer at this time.**

Please talk to your regular doctor about these and other options.

SUBJECT'S RIGHTS

You should not feel obligated to agree to participate. Your questions should be answered clearly and to your satisfaction.

If you decide not to participate, tell the investigators. You will still receive care for your disease and will not lose any benefits to which you would otherwise be entitled.

You will be told of any important new information that is learned during the course of this research study, which might affect your condition or your willingness to continue participation in this study.

CONFIDENTIALITY

Your identity will be kept as confidential as possible as required by law. Except as required by law, you will not be identified by name, social security number, address, telephone number, or any other direct personal identifier. Your research records may be disclosed outside of Stanford, but in this case, you will be identified only by a unique code number. Information about the code will be kept in a secure location and access limited to research study personnel.

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The results of this research study may be presented at scientific or medical meetings or published in scientific journals. However, your identity will not be disclosed.

The purpose of this research study is to obtain data or information on the safety and effectiveness of Lovastatin for reducing the risk of breast cancer; the results will be provided to the sponsor, the Food and Drug Administration and other federal and regulatory agencies as required.

FINANCIAL CONSIDERATIONS

PAYMENT

You will not receive any payment for participating in this study.

COSTS

The sponsor will provide you with Lovastatin free of charge while you are being treated on this study. There will be no charge for the study procedure of breast fine needle aspiration. However, your insurance will be billed for standard studies including mammogram and breast magnetic resonance imaging.

Should Lovastatin become commercially available or approved for this indication during the course of this study, you may be asked to purchase subsequent doses of the agent needed to complete the study.

Taking part in this study may lead to added costs to you or your insurance company. Please ask about any expected added costs or insurance problems.

SPONSORS

The sponsors of this project, the Breast Cancer Research Foundation, the Vadasz Family Foundation and the American Society of Clinical Oncologists are providing financial support and/or material for this study.

CONTACT INFORMATION

If you need to change your appointment, please contact Meredith Mills at 650-724-5223.

If you have any questions about this **research study**, its procedures, risks and benefits, or alternative courses of treatment, you should ask the investigators, Dr. James Ford and Dr. Allison Kurian. You may contact him or her at 650-724-5223. If you have any additional questions later, Dr. James Ford and Dr. Allison Kurian will be happy to answer them.

If you think you have experienced a **research-related injury** call Dr. James Ford and Dr. Allison Kurian at 650-724-5223.

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If you have any questions about your rights as a research subject, you may contact the Administrative Panel on Human Subjects in Medical Research at (650) 723-5244.

COMPENSATION

All forms of medical diagnosis and treatment -- whether routine or experimental -- involve some risk of injury. In spite of all precautions, you might develop medical complications from participating in this study. If such complications arise, the investigators and the research study staff will assist you in obtaining appropriate medical treatment but this study does not provide financial assistance for additional medical or other costs. Additionally, Stanford is not responsible for research and medical care by other institutions or personnel participating in this study. You do not waive any liability rights for personal injury by signing this form. For further information, please call (650) 723-5244 or write the Administrative Panel on Human Subjects in Medical Research, Administrative Panels Office, Stanford University, Stanford, CA 94305-5401. In addition, if you are not satisfied with the manner in which this study is being conducted or if you have any questions concerning your rights as a research study subject, please contact the Human Subjects Office at the same address and telephone number.

EXPERIMENTAL SUBJECT'S BILL OF RIGHTS:

As a human subject you have the following rights. These rights include but are not limited to the subject's right to:

- 1) Be informed of the nature and purpose of the experiment;
- 2) Be given an explanation of the procedures to be followed in the medical experiment, and any drug or device to be utilized;
- 3) Be given a description of any attendant discomforts and risks reasonably to be expected;
- 4) Be given an explanation of any benefits to the subject reasonably to be expected, if applicable;
- 5) Be given a disclosure of any appropriate alternatives, drugs or devices that might be advantageous to the subject, including their relative risks and benefits;
- 6) Be informed of the avenues of medical treatment, if any available to the subject after the experiment if complications should rise;
- 7) Be given an opportunity to ask questions concerning the experiment or the procedures involved;
- 8) Be instructed that consent to participate in the medical experiment may be withdrawn at any time and the subject may discontinue participation without prejudice;
- 9) Be given a copy of the signed and dated consent form; and
- 10) Be given the opportunity to decide to consent or not to consent to a medical experiment without the intervention of any element of force, fraud, deceit, duress, coercion or undue influence on the subject's decision.

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YOUR SIGNATURE INDICATES THAT YOU HAVE READ AND UNDERSTAND THE ABOVE INFORMATION, THAT YOU HAVE DISCUSSED THIS STUDY WITH THE PERSON OBTAINING CONSENT, THAT YOU HAVE DECIDED TO PARTICIPATE BASED ON THE INFORMATION PROVIDED, AND THAT A COPY OF THIS FORM HAS BEEN GIVEN TO YOU.

Signature of Subject

Date

Signature of Legally Authorized Representative

Date

Description of Representative's Authority to Act for Subject

Person Obtaining Consent

I attest that the requirements for informed consent for the medical research project described in this form have been satisfied – that the subject has been provided with the Experimental Subject's Bill of Rights, if appropriate, that I have discussed the research project with the subject and explained to him or her in non-technical terms all of the information contained in this informed consent form, including any risks and adverse reactions that may reasonably be expected to occur. I further certify that I encouraged the subject to ask questions and that all questions asked were answered.

Signature of Person Obtaining Consent

Date

Date of Approval: October 18, 2005

Date of Expiration: October 17, 2006